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INDUCTION OF DRUG-METABOLIZING ENZYMES BY GARLIC AND ALLYL SULFIDE COMPOUNDS VIA ACTIVATION OF CAR AND NRF2

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RUNNING TITLE

Transcription factor activation by garlic.

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Abbreviations

GO, garlic oil; DAS, diallyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide; NQO1, NAD(P)H quinone oxidoreductase 1; CAR, constitutive androstane receptor; Nrf2, nuclear factor E2-related factor2; WKY, Wistar-Kyoto; ARE, antioxidant response element; GST, glutathione-*S*-transferase; UGT, UDP-glucuronosyl transferase; PB, phenobarbital; CO, corn oil; bDNA, branched DNA.

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Abstract

Garlic oil (GO) contains several linear sulfur compounds, including diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS), that induce drug-metabolizing enzymes such as CYP2B and NAD(P)H quinone oxidoreductase1 (NQO1). CYP2B and NQO1 are primarily regulated by constitutive androstane receptor (CAR) and nuclear factor E2-related factor2 (Nrf2) transcription factors, respectively. The purpose of this study was to determine whether GO and its specific constituents induce these two enzymes via CAR and Nrf2 activation. Female Wistar-Kyoto (WKY) rats express little CAR protein and exhibit less induction of CYP2B1/2 than males. GO, DAS and DADS, but not DATS, induced CYP2B1/2 mRNA levels to a greater extent in WKY males than in females, suggesting CAR activation. Conversely, DAS induced NQO1 levels equally in WKY males and females indicating CAR-independent induction in rats. DAS, but not GO, DADS or DATS, induced CYP2B10 mRNA levels 530-fold in wild-type (WT) mice, whereas this induction was attenuated in $CAR^{-/-}$ mice. DAS induced NQO1 in WT and $CAR^{-/-}$ mice equally, suggesting CAR-independent induction in mice. DAS induced NQO1 5-fold in WT mice, whereas induction was completely absent in $Nrf2^{-/-}$ mice, indicating DAS also activates Nrf2. DAS induction of CYP2B10 mRNA was independent of Nrf2 presence or absence. In *in vivo* transcription assays, DAS activated the human *CYP2B6* promoter, and the Antioxidant Response Element (ARE) of the human *NQO1* promoter, respectively. These studies indicate that garlic oil constituents, particularly DAS, activate CAR and Nrf2 to induce drug-metabolizing enzymes.

Introduction

Garlic (*Allium sativum L.*) is an herb consumed in both foodstuffs and dietary supplements. Garlic has known antioxidant activity and has been hypothesized to be beneficial to human health, including cardio-protective and chemotherapeutic properties (Ohaeri and Adoga, 2006;Siddique and Afzal, 2004). Epidemiological studies suggest that high garlic intake hinders development of certain human cancers and can lower the risk of distal colon cancer by 50% (Steinmetz et al., 1994). Given these potential benefits of garlic, dietary supplementation with garlic oil or various types of garlic extracts has increased (Ross et al., 2006).

Gas chromatographic-mass spectral analysis has identified 47 compounds in garlic oil, 18 of which are volatile linear sulfur-containing molecules that account for 94% of garlic oil constituents (Calvo-Gomez et al., 2004). Among the most abundant of these linear sulfur-containing compounds are diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS). DAS can inhibit CYP2E1 activity *in vivo*, and can induce hepatic mRNA levels of CYP1A, 2B, and 3A (Cherrington et al., 2003;Le Bon et al., 2003). Additionally, administration of DADS or DATS results in induction of Phase II and anti-oxidant enzymes such as glutathione-*S*-transferase (GST), NAD(P)H quinone oxidoreductase (NQO1), UDP-glucuronosyl transferase (UGT) and epoxide hydrolase (Fukao et al., 2004;Singh et al., 1998;Wu et al., 2002). However, the mechanism(s) of drug-metabolizing enzyme induction by garlic oil constituents remains unclear.

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The induction of several drug-metabolizing enzymes has been shown to be regulated by the activation of specific transcription factors, including constitutive androstane receptor (CAR) and nuclear factor E2-related factor (Nrf2). These transcription factors act as biosensors for endogenous and xenobiotic chemicals, and respond by increasing drug-metabolizing enzyme levels (Zhang et al., 2004). CYP2B and NQO1 induction is the hallmark of CAR and Nrf2 activation, respectively, and both of these genes are induced by garlic oil constituents.

CAR is best known for its ability to regulate induction of the CYP2B gene family following activation by phenobarbital (PB) and a family of PB-like inducers (Swales and Negishi, 2004). CAR plays a key role in the control of drug metabolism by mediating the induction of many Phase I and II drug-metabolizing enzymes, (such as CYP2B, 2C, 3A, UGT1A1, and GSTA1) as well as drug transporters, including Mrp2 and Oatp4 (Arnold et al., 2004;Huang et al., 2003).

Nrf2 regulates the gene expression of a battery of enzymes that serve to detoxify electrophiles and pro-oxidative stressors (Numazawa and Yoshida, 2004). Activation of Nrf2 results in transcriptional activation of several genes involved in the antioxidant response including: NQO1, NRH:quinone oxidoreductase 2, GSTA1, γ-glutamylcysteine synthetase and heme oxygenase 1 (Chen and Kong, 2004;Hayes and McMahon, 2001;Jaiswal, 2004).

Because the intake of garlic and garlic supplements is prevalent, understanding the mechanisms governing the pharmacological actions of garlic is paramount to predict the potential for garlic and garlic supplements to alter drug metabolism. Previous studies reported that garlic alters the pharmacokinetics of several therapeutic drugs, including the HIV protease inhibitor saquinavir, the analgesic/antipyretic paracetamol, and the anticoagulant warfarin (Izzo and Ernst, 2001;James, 2001). Thus the current study was conducted to determine whether garlic oil and garlic oil constituents, namely DAS, DADS, and DATS, coordinately regulate drug-metabolizing enzymes by activation of the transcription factors CAR and Nrf2.

Materials and Methods

Chemicals. Garlic oil (GO) was obtained from Spectrum Chemicals and Laboratory Equipment (Gardena, CA). DAS, DADS and corn oil were purchased from Sigma-Aldrich, Co. (St. Louis, MO) at the highest purities available. DATS was purchased from LKT Laboratories, Inc. (St. Paul, MN). Phenobarbital was purchased from Mallinckrodt, Inc (Paris, KY). Ketamine HCl and Xylazine injectables were purchased from Associated Medical Supply (Scottsdale, AZ). Luciferin was obtained from Molecular Imaging Products Company (Ann Arbor, MI).

Animals. Male and female Wistar-Kyoto (WKY) rats and male C57BL6/J wild-type (WT) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Male mice homozygous for the targeted mutation of CAR were developed as previously described (Wei et al., 2000) were obtained from Deltagen (San Carlos, CA). $Nrf2^{-/-}$ mice were generated as previously described (Itoh et al., 1997). $CAR^{-/-}$ mice used in the present studies were bred on a mixed SVJ129/C57BL/6 background, whereas $Nrf2^{-/-}$ mice were bred on a C57BL/6 background.

Male and female WKY (n=5) rats were administered phenobarbital (80 mg/kg, ip), GO (300 mg/kg, po), DAS (500 mg/kg, po), DADS (200 mg/kg, po), DATS (80 mg/kg, po) or corn oil. Male WT, $CAR^{-/-}$ and $Nrf2^{-/-}$ mice were administered GO (175 mg/kg, po), DAS (500 mg/kg, po), DADS (80 mg/kg, po), DATS (80 mg/kg, po) or corn oil. All treatments were carried out for four days at a volume of 5 ml/kg and total RNA was prepared from livers.

All animals were acclimated for at least one week prior to experiments and allowed water and standard chow *ad libitum*. Housing and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals as determined by the U.S. National Institutes of Health.

Total RNA Isolation: Total RNA was isolated using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) as per the manufacturer's protocol. RNA concentrations were determined by UV spectrophotometry, and integrity was examined by ethidium bromide staining after agarose gel electrophoresis.

Branched DNA assay. Probe sets for rat CYP2B1/2, rat NQO1, mouse CAR, CYP2B10, mouse Nrf2 and mouse NQO1 were used as previously described (Cheng et al., 2005;Cherrington et al., 2002;Cherrington et al., 2003;Hartley and Klaassen, 2000). Specific oligonucleotide probes were diluted in lysis buffer supplied in the Quantigene™ HV Signal Amplification Kit (Panomics, Inc., Freemont, CA). All reagents for analysis (*i.e.,* lysis buffer, capture hybridization buffer, amplifier/label probe buffer, and substrate solution) were supplied in the Quantigene Discovery Kit. Total RNA (1 µg/µl; 10 µl) was added to each well of a 96-well plate containing capture hybridization buffer and 50 µl of each diluted probe set. Total RNA was allowed to hybridize to each probe set overnight at 53°C. Subsequent hybridization steps were carried out as per the manufacturer's protocol, and luminescence was quantified with a Quantiplex™ 320 bDNA luminometer interfaced with Quantiplex™ Data Management Software Version 5.02 in 96-well plates.

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In vivo luciferase assay. A human *CYP2B6* promoter construct containing a 1.7-kb fragment which maintains the core promoter (+39/-364) and the distal enhancer region (-1461/-2013), including the phenobarbital-responsive element cloned into pGL3-basic, was obtained from Dr. Richard Kim (Vanderbilt University, Nashville, TN). An AREx5 promoter construct was obtained from Dr. David Ross (University of Colorado Health Sciences Center) and contains five consecutive copies of the human ARE sequence found in the human *NQO1* promoter cloned into a RSV180-luciferase reporter. C57BL6/J mice were age- and weight-matched. Mice were given a rapid (5-s) tail-vein injection of naked plasmid DNA, either 10 µg human *CYP2B6*-luciferase or 3 µg human AREx5-luciferase reporter constructs, in sterile saline at a volume equal to 10% body weight. Following an 18 h recovery, mice were anesthetized with ketamine/xylazine and injected with 0.07 µl of 25 mg/ml D-luciferin in saline vehicle (Molecular Imagining Products Company, Ann Arbor, MI) five min prior to imaging. A VersArray 1300B camera (Princeton Instruments, Trenton, NJ) thermoelectrically cooled to -100ºC was used to image mice. Images were obtained using Win View 32 software (Princeton Instruments, Trenton, NJ) in gray-scale, and pseudo-color maps were created with the Win View 32 program. Color maps were superimposed over the light image of the mouse using Adobe® Photoshop® CS2 software. This image was considered time 0 h. Mice (n=4) were then administered GO (300 mg/kg, po), DAS (500 mg/kg, po), DADS (200 mg/kg, po), DATS (80 mg/kg, po) or corn oil in a volume of 5 ml/kg. Images were repeated at 12 and 24 h for the human AREx5- or human *CYP2B6*-transfected mice, respectively. Quantification of promoter induction was determined by densitometry using SimplePCI software (Compix Inc., Sewickley, PA).

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Statistics. Statistical significance was determined by one-way ANOVA followed by a Newman-Keuls post-hoc test between all groups. ($p \leq 0.05$).

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Results

CYP2B1/2 *and* NQO1 *induction in Wistar-Kyoto (WKY) rats.* WKY rats display a gender-specific dimorphism where female rats express less CAR protein than males. As a result, female WKY rats administered a CAR-activating xenobiotic, such as phenobarbital, show less hepatic CYP2B1/2 mRNA induction than male WKY rats. In this study, WKY rats were used to determine whether garlic oil (GO) or its three major linear sulfur compounds (DAS, DADS and DATS) induce hepatic CYP2B1/2 and NQO1 via CAR activation.

Figure 1A shows CYP2B1/2 mRNA levels in male and female WKY livers following 4-day treatment with corn oil (CO) vehicle, phenobarbital (PB, positive control for CAR activation), GO and its constituents. PB increased CYP2B1/2 mRNA levels in male 5.6-fold higher than in female WKY rats. Similarly, GO, DAS and DADS induced male WKY rat CYP2B1/2 mRNA 8.5-, 1.3- and 9.2-fold, respectively, when compared to WKY females. DATS treatment failed to induce CYP2B1/2 in either male or female WKY rats.

Figure 1B, Hepatic NQO1 mRNA levels were not significantly induced by GO, DADS or DATS in male or female WKY rats. However, DAS induced NQO1 mRNA levels approximately 7.5-fold in both male and female WKY rats.

CYP2B10 *and* NQO1 *induction in CAR$^{-/-}$ mice.* To definitively determine whether GO and its constituents induce CYP2B10 via CAR activation, levels of hepatic CYP2B10 mRNA were examined in wild-type (WT) and CAR$^{-/-}$ mice following treatment with GO,

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DAS, DADS and DATS. GO tended to increase levels of CYP2B10 mRNA in WT and $CAR^{-/-}$ mice; however, this induction was not statistically significant (Figure 2A). DAS induced CYP2B10 mRNA levels 530-fold in WT mice, but not at all in $CAR^{-/-}$ mice. DADS and DATS did not affect hepatic CYP2B10 mRNA levels in WT or $CAR^{-/-}$ mice.

Hepatic NQO1 levels were also determined in WT and $CAR^{-/-}$ mice administered GO, DAS, DADS and DATS (Figure 2B). GO tended to increase NQO1 mRNA levels in WT mice, although this induction did not reach statistical significance from control, whereas GO induced hepatic NQO1 mRNA levels approximately 10-fold in $CAR^{-/-}$ mice. DAS increased NQO1 mRNA levels similarly in WT and $CAR^{-/-}$ mice (31- and 32-fold, respectively). DADS tended to increase the levels of NQO1 mRNA in WT and $CAR^{-/-}$ mice (8- and 9-fold, respectively), but theses increases were not statistically significant from control. Similarly, DATS tended to increased NQO1 mRNA levels by 9- and 6-fold in WT and $CAR^{-/-}$ mice, respectively, but neither group was statistically significant from control. To ensure that Nrf2 expression was not altered by the deletion of the CAR gene, Nrf2 mRNA levels were determined, and found to be unchanged in WT and $CAR^{-/-}$ mice (data not shown).

CYP2B10 *and* NQO1 *mRNA induction in* $Nrf2^{-/-}$ *mice*. To obtain further evidence on whether GO or its constituents induce hepatic drug-metabolizing enzymes via Nrf2 and/or CAR, NQO1 and hepatic CYP2B10 mRNA levels were determined in WT and $Nrf2^{-/-}$ mice. Figure 3A indicates the induction of CYP2B10 in WT and $Nrf2^{-/-}$ mice following treatment with GO, DAS, DADS and DATS. Surprisingly, GO failed to induce

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CYP2B10 in WT mice, whereas it produced a 37-fold induction of mRNA levels in Nrf2$^{-/-}$ mice. Mice treated with DAS displayed a robust induction of CYP2B10 that was not dependent on the presence or absence of Nrf2. DADS and DATS treatment did not induce hepatic CYP2B10 in WT or Nrf2$^{-/-}$ animals. To ensure that CAR expression was not altered by the deletion of the Nrf2 gene, CAR mRNA levels were determined, and there was no difference between WT and CAR$^{-/-}$ mice (data not shown).

Figure 3B shows the effects of GO and its constituents on hepatic NQO1 induction in WT and Nrf2$^{-/-}$ mice. GO induced NQO1 mRNA levels 12- and 37-fold in WT and Nrf2$^{-/-}$ mice, respectively. However, this induction was only statistically significant in Nrf2$^{-/-}$ mice. The 9-fold induction of NQO1 observed with DAS in WT mice was completely absent in Nrf2$^{-/-}$ mice, indicating that this induction is mediated via Nrf2. DADS treatment tended to increase NQO1 mRNA levels in WT and Nrf2$^{-/-}$ mice, but theses increases were not statistically significant. DATS increased NQO1 mRNA levels 4-fold in WT mice, however, this increase was not significantly different from Nrf2$^{-/-}$ mice.

Human CYP2B6 and AREx5 promoter activity in vivo. Figure 4 shows the effect of garlic oil, DAS, DADS and DATS treatment on the human *CYP2B6*-luciferase reporter construct containing the CAR-specific binding element NR1, in transiently-transfected mice. DAS robustly activated transcription of the human CYP2B6 promoter 24 h after administration, compared to luciferase activity observed at 0 h imaging. Densitometry of images in Figure 4 showed that DAS caused a 5-fold increase in transcriptional

activation of the human *CYP2B6* promoter, whereas CO, GO, DADS and DATS had no effect.

Figure 5 demonstrates transcriptional activation of an AREx5-luciferase reporter construct by GO and its constituents. Because induction of mouse NQO1 is not as marked as CYP2B10, we used a human AREx5-luciferase reporter to increase the response of the promoter to Nrf2 binding. Furthermore, Nrf2 activation and nuclear translocation occurs more rapidly than does CAR. Preliminary experiments demonstrated that optimal transcriptional activation occurs at 12 h following administration of the known Nrf2 inducer Oltipraz (data not shown). DAS resulted in a robust increase in AREx5 transcriptional activation seen in images of mice treated by DAS. Quantification by densitometry revealed that DAS increased transcriptional activation of the human AREx5 8-fold. However, GO, DADS and DATS administration resulted in little to no increase in transcriptional activation of the human AREx5-luciferase reporter construct when compared to 0 h images.

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Discussion

Previous studies have shown the ability of DAS, DADS and DATS to alter levels of hepatic drug-metabolizing enzymes including CYPs, EH, GST and NQO1 both *in vitro* and *in vivo* (Chen et al., 2004;Cherrington et al., 2003;Zhang et al., 2006). Because several of these enzymes fall into specific gene batteries regulated by CAR and Nrf2, the goal of the present study was to determine whether garlic oil (GO) and its constituents induce CYP2B and NQO1 via activation of these transcription factors.

Our results demonstrate for the first time that GO and DADS significantly induce CYP2B1/2 in rats, and confirm DAS induction of CYP2B1/2 observed in previous reports (Lii et al., 2006). Because CAR protein expression in Wistar-Kyoto rats is much lower in female than in male livers (Yoshinari et al., 2001), the poor induction of CYP2B1/2 after GO, DAS and DADS in female rats when compared to males strongly suggests that CAR is involved in the hepatic induction of this drug-metabolizing enzyme. Our lab has previously shown that DAS treatment results in a greater induction of CYP2B1/2 mRNA levels in male WKY rats than in females (Cherrington et al., 2003), similar to the current study. The observation that the sex difference in CYP2B1/2 induction by DAS is smaller than that observed by GO and DADS is curious and may be indicative of the multiple components involved in the CAR-mediated induction of CYP2B1/2. How CAR levels affect either Mrf2 or other transcription factors is the subject of ongoing research.

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DAS also induces CYP2B10 in mice (Cheng et al., 2005). Furthermore, the CAR-RXRα heterodimer is important in basal CYP2B10 transcription, as demonstrated by studies using hepatic RXRα$^{-/-}$ mice (Cherrington et al., 2003). We have previously shown that DAS induction of CYP2B10 is significantly reduced in RXRα$^{-/-}$ mice, suggesting that CYP2B10 induction following DAS administration is CAR-dependent, because of the loss of the obligate herterodimerizing partner (Cherrington et al., 2003). The robust induction of CYP2B10 caused by DAS in WT mice was completely absent in CAR$^{-/-}$ mice and indicates that CYP2B10 induction is CAR-dependent. In addition, DAS was shown to activate a human *CYP2B6* promoter-reporter construct containing the NR1 CAR-binding element in transiently-transfected mice. These results suggest that DAS activation of CAR is a mechanism of CYP2B induction conserved between rats and mice.

DAS significantly increased NQO1 mRNA levels in both male and female WKY rats (Figure 1B), as well as wild-type and CAR$^{-/-}$ mice (Figure 2B). Together these results suggest that DAS activates NQO1 via a CAR-independent mechanism. Previous studies have noted that DAS causes a 3-fold increase in hepatic GST activity in mice, another antioxidant gene regulated by Nrf2 (Srivastava et al. 1997). Additionally, they reported 3.2- and 4.4-fold inductions of hepatic GST mRNA following DADS and DATS treatment, respectively. In contrast, Wu et al. reported that while DADS and DATS increased levels of NQO1 *in vitro* , DAS failed to induce these enzymes (Wu et al., 2004). In the current study, definitive evidence that Nrf2 is involved in the induction of NQO1 by GO and its constituents was determined using Nrf2$^{-/-}$ mice. DAS produced a

6-fold increase in NQO1 mRNA levels in WT mice, which was almost completely prevented in Nrf2$^{-/-}$ mice. In addition, DAS administration to transiently-transfected mice was shown to activate a human ARE x5-luciferase reporter, suggesting the possibility that DAS might also induce NQO1 in humans.

DAS has been shown to cause nuclear accumulation of CAR and binding to the CAR specific NR1 element in the promoter of CYP2B1/2 in rats (Zhang et al., 2006). However, DAS activation of CAR has not been examined in mice and Nrf2 activation has only been implied *in vitro* with varying results. Two studies have examined the possibility that DAS activates Nrf2 and thus induces antioxidant genes in HepG2 cells. One study demonstrated that DATS was the most potent ARE inducer among the three garlic constituents examined in this study, whereas DAS had no effect on ARE transcriptional activity (Chen et al., 2004). Contrary to the current findings, another study noted significant increases in protein expression, nuclear translocation, and DNA-binding of Nrf2, respectively, in HepG2 cells following treatment with DAS (Gong et al., 2004).

Several xenobiotics specifically activate either CAR (phenobarbital, chlorpromazine, phenytoin) or Nrf2 (sulforaphane and butylated hydroxyanisole). The prototypical CAR activator, phenobarbital, has been shown to induce Nrf2-regulated genes including NQO1 in addition to CYP2B10 in the mouse (Slitt et al., 2006). We have further demonstrated that the Nrf2 activator ethoxyquin also increases mRNA levels of CYP2B1/2 in rats, suggesting that this xenobiotic may likewise activate CAR in addition

to Nrf2 (Cherrington et al., 2003). Recently, *trans*-stilbene oxide has been shown to activate both CAR and Nrf2 (Slitt et al., 2006). These results led Slitt et al. to hypothesize that cross-talk between the CAR and Nrf2 activation pathways could occur with *trans*-stilbene oxide. The fact that DAS can activate these same transcription factors in both mice and rats suggests the possibility of cross-talk between CAR and Nrf2. Importantly, this may explain the unexpected induction of CYP2B10 by garlic oil in $Nrf2^{-/-}$ mice where induction was not seen in WT mice. Additionally, the observed induction of NQO1 in both $CAR^{-/-}$ and $Nrf2^{-/-}$ mice by GO underscores the complexity of the several components that make up GO. Whereas the concept of cross-talk between CAR and Nrf2 activation pathways has been hypothesized (Slitt, 2006), further studies are necessary to determine the nature and biochemical consequences of this potential mechanism

DAS, DADS, and DATS have been identified as three major constituents in garlic oil (Wu et al., 2004). Because these chemicals have all been documented to affect transcriptional regulation of hepatic phase I and phase II drug-metabolizing enzymes, it is reasonable to expect that garlic oil would have similar effects. Although GO produced significant induction of CYP2B1/2 in WKY rats (Figure 1A), induction of CYP2B10 was not observed in WT mice following a four day induction study. This is almost certainly due to the dose-limiting toxicity observed in mice treated with GO in the current study. While rats were able to tolerate four consecutive days of garlic oil (300 mg/kg, po), this dose was not tolerated in mice. The GO dose was therefore lowered to 175 mg/kg to complete the four day induction studies in mice. A similar situation was observed in

mice treated with DADS. Due to toxicity observed with 200 mg/kg DADS in mice, a dose of 80 mg/kg DADS was used to complete the four day induction studies. The apparent lack of mouse CYP2B10 induction following GO and DADS could be associated with this decrease in dose concentration. It is also noteworthy that $Nrf2^{-/-}$ mice were particularly susceptible to DATS-induced lethality, an observation not noted in WT or $CAR^{-/-}$ mice. Consistent with previous studies, it is likely that Nrf2 plays a role in preventing xenobiotic toxicity of compounds via induction of detoxification and antioxidant enzymes (Jaiswal, 2004). Unlike the four day dosing studies, designed to determine maximal induction of mRNA levels following administration of an inducer, the *in vivo* transcription assay is designed to measure activation of transcription. We have previously shown that a single dose of *trans*-stilbene oxide (Slitt et al., 2006) results in a robust transcriptional activation of the human CYP2B-luciferase reporter in this assay. Single doses of garlic oil (200 mg/kg, po) and DADS (200 mg/kg, po) used in the *in vivo* transcription assay studies were better tolerated than in the four day induction studies.

Preclinical evidence continues to elucidate the antibacterial, antithrombotic and chemotherapeutic properties of fresh garlic extracts, aged garlic, garlic oil, and a number of specific organosulfur compounds generated by processing garlic (Ariga and Seki, 2006;Milner, 2006;Sengupta et al., 2006). As usage of garlic supplements increases, it is important to understand the biological effects of such intake. The present data indicate that a specific constituent of garlic oil, DAS, activates CAR and Nrf2, thereby altering drug metabolism. Thus the potential for herb-drug interactions

with garlic and its organosulfur constituents exists, and garlic intake may need to be taken into consideration in the clinical setting.

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Footnotes

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Figure Legends

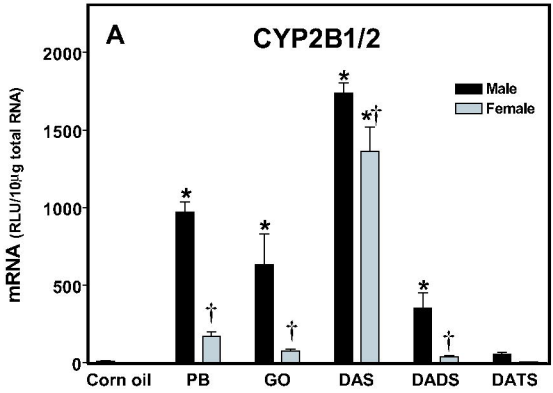
Figure 1. Hepatic induction of CYP2B1/2 (**A**) and NQO1 (**B**) in male and female Wistar Kyoto rats following treatment with garlic oil and its constituents. Total hepatic RNA from male and female WKY rats (n=5) treated with phenobarbital (PB), garlic oil (GO), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) or corn oil (CO) vehicle was analyzed for mRNA levels of each gene. Data expressed as relative light units (RLU) ± standard error of mean. Significance indicated from control (*) or between genders (†) ($p \leq 0.05$).

Figure 2. Hepatic induction of CYP2B10 (**A**) and NQO1 (**B**) in wild-type and $CAR^{-/-}$ mice following treatment with garlic oil and its constituents. Total hepatic RNA from WT and $CAR^{-/-}$ mice (n=5) treated with GO, DAS, DADS, DATS or CO vehicle was analyzed for mRNA levels of each gene. Data expressed as relative light units (RLU) ± standard error of mean. Significance indicated from control (*) or between WT and knockout (†) ($p \leq 0.05$).

Figure 3. Hepatic induction of CYP2B10 (**A**) and NQO1 (**B**) in wild-type and $Nrf2^{-/-}$ mice following treatment with garlic oil and its constituents. Total hepatic RNA from WT and $Nrf2^{-/-}$ mice (n=5) treated with GO, DAS, DADS, DATS or CO vehicle was analyzed for CYP2B10 mRNA levels. Data expressed as relative light units (RLU) ± standard error of mean. Significance indicated from control (*) or between WT and knockout (†) ($p \leq 0.05$).

Figure 4. Transcriptional activation of the human *CYP2B6* promoter *in vivo*. Mice were hydrodynamically transfected with a 1.7-kb fragment of the human CYP2B6 promoter, which includes the distal enhancer region, inserted into a firefly luciferase reporter vector. At T=0, transfection efficiency was assessed by imaging luciferase intensity *in vivo* and then mice were given a single dose of GO, DAS, DADS, DATS or CO vehicle. 24 h post treatment, luciferase intensity was imaged a second time and compared to T=0 images to determine promoter activation. Luciferase intensity was quantified by densitometry using SimplePCI software. Data are expressed as mean fold induction of each promoter ± standard error of mean. (*) indicates statistical significance from CO group ($p \leq 0.05$, n=4).

Figure 5. Transcriptional activation of the human antioxidant response element (ARE) promoter *in vivo*. Mice were hydrodynamically transfected with 5x multimer of the human ARE sequence inserted into a firefly luciferase reporter vector. At T=0, transfection efficiency was assessed by imaging luciferase intensity *in vivo* and then mice were given a single dose of GO, DAS, DADS, DATS or CO vehicle. 12 h post treatment, luciferase intensity was imaged a second time and compared to T=0 images to determine promoter activation. Luciferase intensity was quantified by densitometry using SimplePCI software. Data are expressed as mean fold induction of each promoter ± standard error of mean. (*) indicates statistical significance from CO group ($p \leq 0.05$, n=4).


A
CYP2B1/2
mRNA (RLU/10μg total RNA)
2000
1500
1000
500
0
Male
Female
*
*†
*
†
Corn oil
PB
GO
DAS
DADS
DATS

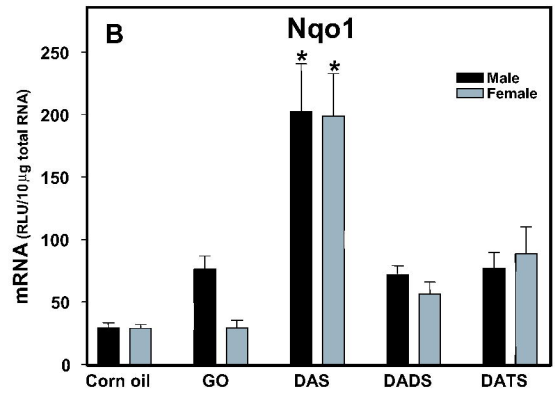
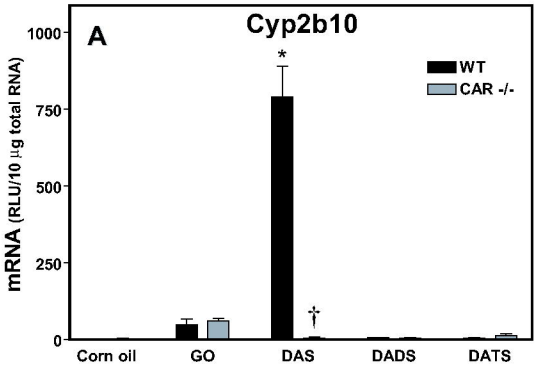

B
Nqo1
mRNA (RLU/10μg total RNA)
250
200
150
100
50
0
Male
Female
*
*
Corn oil
GO
DAS
DADS
DATS

Figure 1


A
Cyp2b10
mRNA (RLU/10 μg total RNA)
1000
750
500
250
0
WT
CAR -/-
*
†
Corn oil
GO
DAS
DADS
DATS

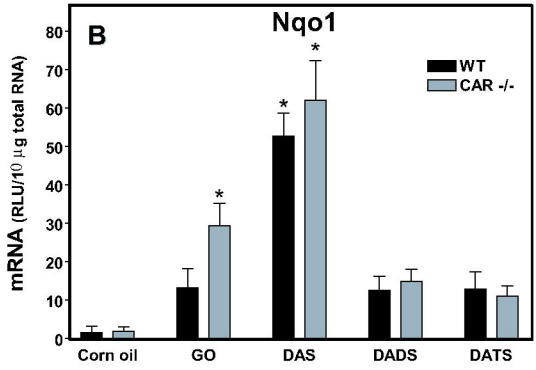

B
Nqo1
mRNA (RLU/10 μg total RNA)
80
70
60
50
40
30
20
10
0
WT
CAR -/-
*
Corn oil
GO
DAS
DADS
DATS

Figure 2

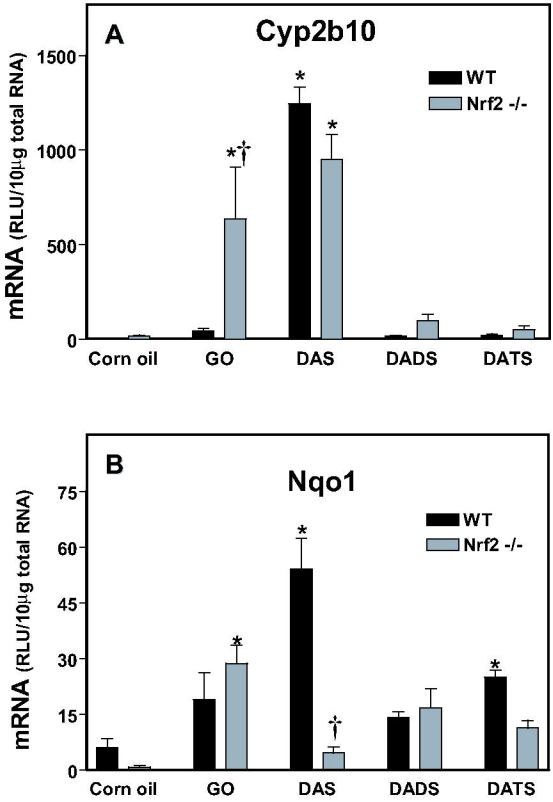
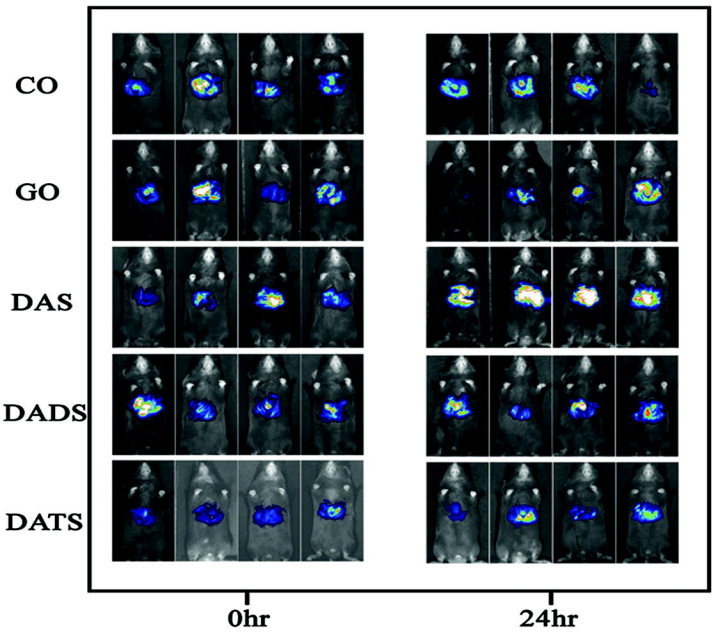

A
Cyp2b10
mRNA (RLU/10μg total RNA)
1500
1000
500
0
WT
Nrf2 -/-
*
*†
*
Corn oil
GO
DAS
DADS
DATS
B
Nqo1
mRNA (RLU/10μg total RNA)
75
60
45
30
15
0
WT
Nrf2 -/-
*
*
†
*
Corn oil
GO
DAS
DADS
DATS

Figure 3

Figure 4


CYP2B6
CO
GO
DAS
DADS
DATS
0hr
24hr

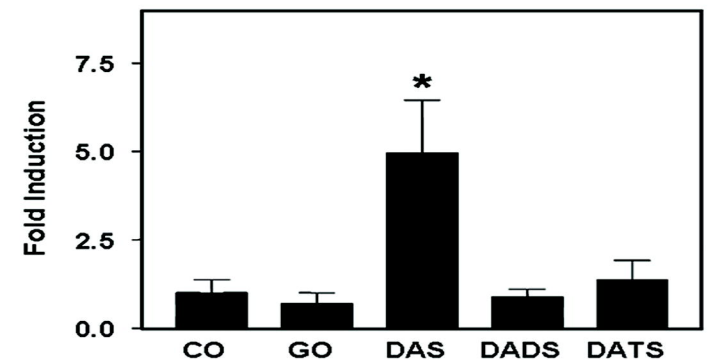
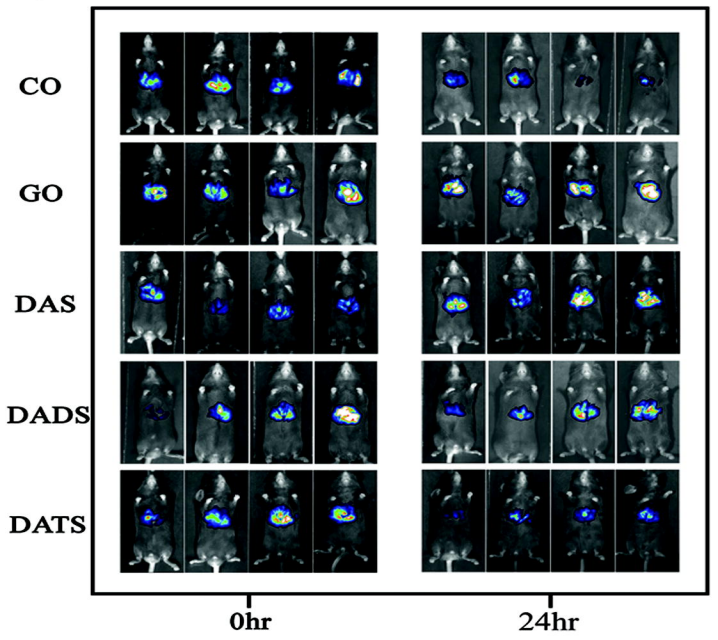
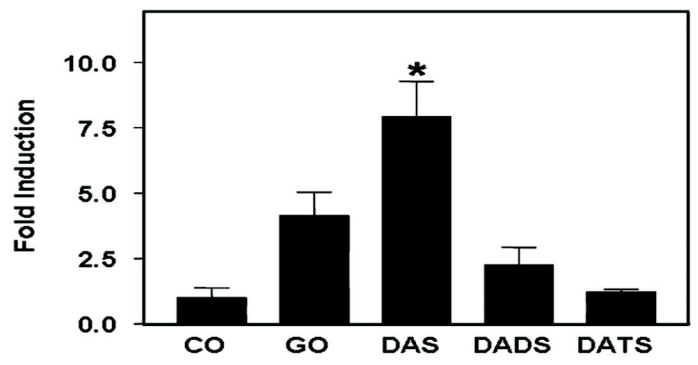

Fold Induction
7.5
5.0
2.5
0.0
*
CO
GO
DAS
DADS
DATS

Figure 5


ARE×5
CO
GO
DAS
DADS
DATS
0hr
24hr


Fold Induction
10.0
7.5
5.0
2.5
0.0
*
CO
GO
DAS
DADS
DATS